Mapping of the cytochrome *c* binding site on cytochrome *c* oxidase

Roberto Bisson, Guy C.M. Steffens*, Roderick A. Capaldi+ and Gerhard Buse*

*CNR Center for Physiology of Mitochondria and Laboratory of Molecular Biology and Pathology, Instituto di Patologia Generale, Università di Padova, via Loredan 16, 35100 Padova, Italy, *Abteilung Physiologische Chemie der RWTH Aachen, Schneebergweg 211, 5100 Aachen, FRG* and *+Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA*

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1. INTRODUCTION

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal enzyme of the mitochondria respiratory chain catalysing electron transfer from cytochrome *c* to molecular oxygen [1,2]. The molecular mechanism of this process is still not understood. At present, little is known about such important structural features as the position of the prosthetic groups or the location and characteristics of the cytochrome *c* binding sites in the cytochrome *c* oxidase complex [3]. The aim of this paper is to provide information about this latter problem as part of a more general effort of our laboratories to elucidate the relationship between structure and functions of cytochrome *c* oxidase.

We have shown that lysine 13, one of the amino acid residues involved in binding cytochrome *c* to reductases and oxidases [4], can be selectively modified with a photoreactive arylazido group [5]. This cytochrome *c* derivative is activated upon illumination to form a covalent enzyme–substrate complex. In the case of cytochrome *c* oxidase, binding has been shown to occur in the high affinity binding site for cytochrome *c* and involve subunit II [5,6]. The work presented here locates the site of interaction of the lysine 13-modified arylazido cytochrome *c* to the C-terminal part of this polypeptide at or close to histidine 161.

2. MATERIALS AND METHODS

Horse heart cytochrome *c* modified with an arylazido group at lysine 13 was prepared and purified as in [5]. Cytochrome *c* oxidase was isolated from beef heart as in [7], the final dialysis step was omitted. The activity, measured polarographically in 0.5% Tween 80, 50 mM phosphate buffer (pH 7.4) ranged from 130–170 mol cytochrome $c \cdot s^{-1} \cdot$ mol aa_3^{-1}.

The covalent enzyme–substrate complex was obtained essentially as in [5,6]. Briefly, 150 mg oxidase were diluted into 250 ml deaerated 1 mM Tris–EDTA, 0.3% Tween 80 (pH 7.0). Arylazido cytochrome *c* (25 mg) diluted in the same buffer (40 mg/ml), was added and the solution irradiated with a 100 W mineral lamp (Ultra-Violet Products, San Gabriel CA) through a glass filter, at 0°C for 60 min under stirring. Then the solution was rapidly filtered on an Amberlite CG 50 layer to take out unreacted cytochrome *c*. The cycle of reaction was repeated by adding 15 mg fresh cytochrome *c* derivative. This led to 70–80% inhibition of cytochrome *c* oxidase activity. The photolabeled cytochrome *c* oxidase was recovered by ultra-centrifugation, dissolved in 3% SDS and chromatographed on Bio-Gel P100 in the same medium. The covalent cytochrome *c*–subunit II complex was passed through a Bio-Gel P6 column run with 0.01% SDS and lyophilized.

Tryptic digestion was performed with TPCK trypsin (Merck, Darmstadt) at 37°C overnight in 0.1% NH_4HCO_3 buffer (pH 8.2) in the presence of 0.15% SDS. The enzyme substrate ratio was 1:50. The peptide mixture was lyophilized and dissolved in ethanol:88% formic acid (7:3, v/v) and subsequently chromatographed on Sephadex LH 60 as in [8].

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Thin-layer chromatography was performed on cellulose plates (MN 300-25, Macherey and Nagel, Duren), using a solvent system of pyridine:*n*-butanol:acetic acid:water (40:60:12:48, by vol.).

Amino acid analysis was done as in [9]. Sequencing was performed automatically as in [10] with a Beckman Sequencer 890C. Conversion of the anilinothiazolinones was done as in [11]. Identification of the phenylthiohydantoin amino acids was done by HPLC on a Spectra Physics SP 8000 liquid chromatograph, using a Zorbax ODS column (0.46 × 25 cm, Dupont de Nemours).

3. RESULTS

Purification of the covalent subunit II–cytochrome *c* complex, obtained by photoaffinity labeling of cytochrome *c* oxidase with arylazidocytochrome *c*, was performed on a Bio Gel P100 column in 3% SDS (fig.1). The red, heme containing product (24 + 12.5 = 36.5 kM_r) comigrated with subunit I. Rechromatography did not increase purification but rather led to loss of protein due to aggregation. Thus, fractions containing the covalent complex were pooled and the impure product used directly for fragmentation after most of the SDS was removed by gel chromatography on Bio-Gel P6 in 0.01% SDS. Tryptic cleavage was performed in the presence of 0.15% SDS as in section 2.

The lyophilized peptide mixture was dissolved in ethanol:88% formic acid (7:3, v/v) and chromatographed on Sephadex LH 60 [8] as shown in fig.2. The elution profile was complicated by the presence of fragments coming from subunit I, subunit III (a portion of which runs as a dimer and coelutes with I) as well as fragments from the cytochrome *c*–subunit II complex. However, only 4 heme containing peaks were detected and one of these eluted at the exclusion volume and contains uncleaved product. Much of the subunit I and III, which are only poorly digested by trypsin, were present in the void volume. The other 3 heme containing peaks are labeled

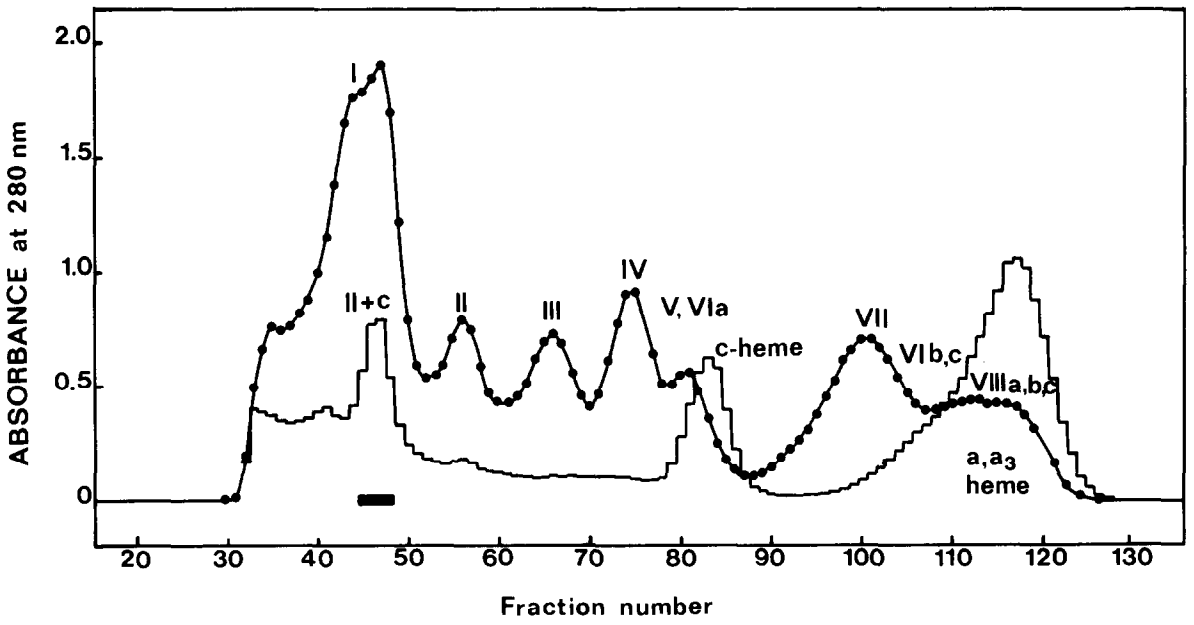

Fig.1. Separation of the cytochrome *c* oxidase polypeptides photolabelled with arylazido-cytochrome *c* enzyme–substrate covalent complex (100 mg) were dissolved in 3% SDS and chromatographed with the same solvent on a Bio Gel P100 (minus 400 mesh) column (5 × 30 cm). The solid line shows the protein elution profile of the oxidase after photolabelling with the cytochrome *c* derivative. The barographs and the horizontal bar, indicate the heme distribution and the fractions pooled, respectively. Nomenclature was taken from [11].

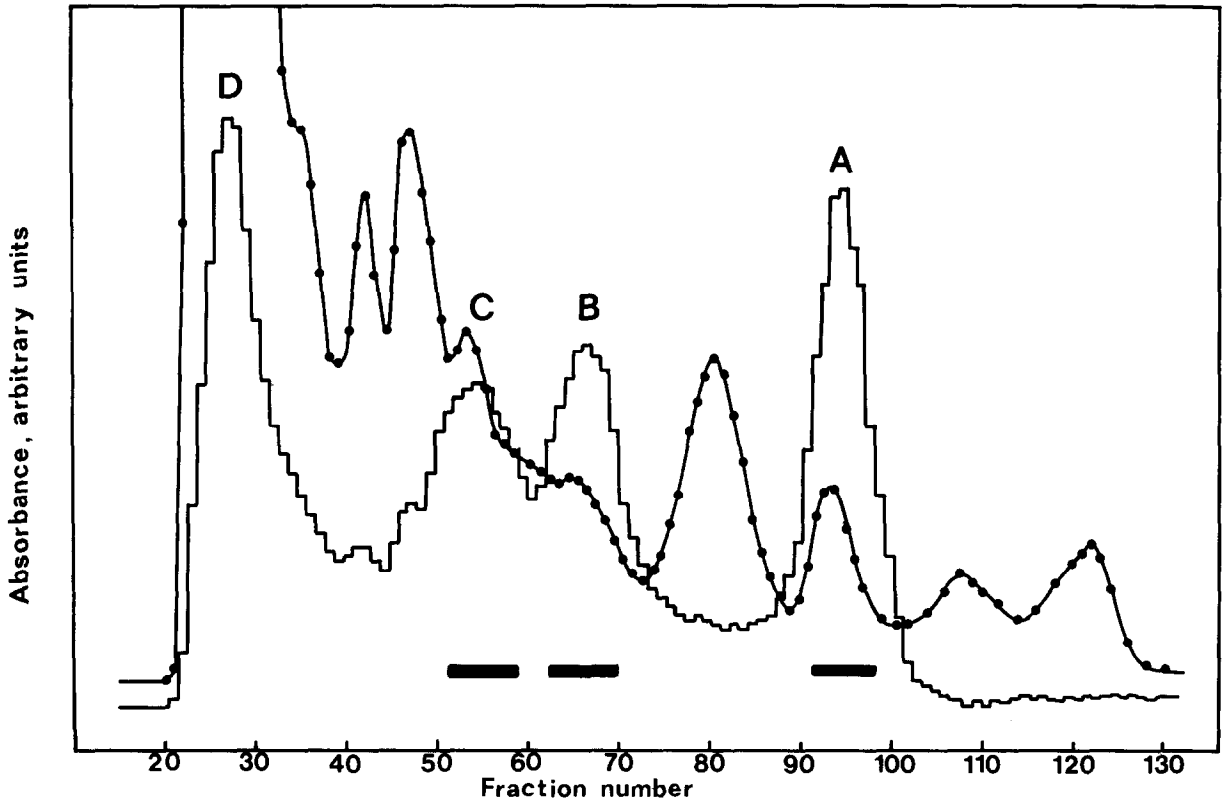

Fig.2. Separation of tryptic fragments of subunit II–cytochrome *c* covalent complex. The pooled fractions containing the photo-crosslinked polypeptides, indicated with a horizontal bar in fig.1, were dried and the protein digested with TPCK-trypsin as in section 2. Hydrophobic chromatography as in [8] was done on a Sephadex LH 60 column (3 × 120 cm) run with ethanol:88% formic acid (7:3, v/v). Fractions of 5 ml were collected. The solid line and the barographs in the figure, show the elution profile and the heme distribution respectively. Horizontal bars indicate the fractions pooled.

A, B, and C in fig.2. Peak A being the smallest, was most likely to be a limit cleavage product and was therefore analysed first.

The peak was first subjected to thin layer chromatography in a pyridine:*n*-butanol:acetic acid: water mixture. This solvent system separates mainly on the basis of hydrophobicity and the migration of fragments is therefore greatly influenced by the bound heme. The fraction was resolved into a major, broad and heme-containing spot and 2 minor colorless spots of protein. The heme-containing material was collected and sequenced through 15 cycles as shown in fig.3. Two sequences were present, one beginning Met–Leu–Val coming from subunit II and generated by cleavage of the arginyl-bond of Arg 152, the other beginning Ile–Phe–Val coming from cytochrome *c* (cleavage of the lysyl bond of Lys 8). Surprisingly, the two sequences were in unequal proportions. This is probably because there are actually 2 crosslinked products present, one involving cytochrome *c* 9–22 + subunit II 152–178; the other cytochrome *c* 1–22 subunit II 152–178. The N-terminus of cytochrome *c* is blocked and thus the fragment 1–22 would not show up in the sequencing steps. The amino acid analysis of fraction A is close to that of a 1:1 mixture of the 2 products above (not shown). Moreover, the broadness of the heme-containing spot is consistent with the cytochrome *c* 1–22–subunit II 152–178 crosslinked product still copurifying with the cytochrome *c* 9–22–subunit II 152–178 product through the thin-layer chromatography.

The yield of amino acids from the cytochrome *c*

Table 1

Conserved amino acids in the sequenced subunit II fragment of beef heart cytochrome *c* oxidase crosslinked to cytochrome *c* lysine 13

		158				161			Ref.
Beef	... Ser	Glu	Asp	Val	Leu	His	Ser	...	[12]
Human	... Ser	Gln	Asp	Val	Leu	His	Ser	...	[17]
Yeast	... Ala	Ala	Asp	Val	Ile	His	Asp	...	[18]
Maize	... Pro	Ala	Asp	Val	Pro	His	Ser	...	[19]

Sequences are aligned to beef heart subunit II

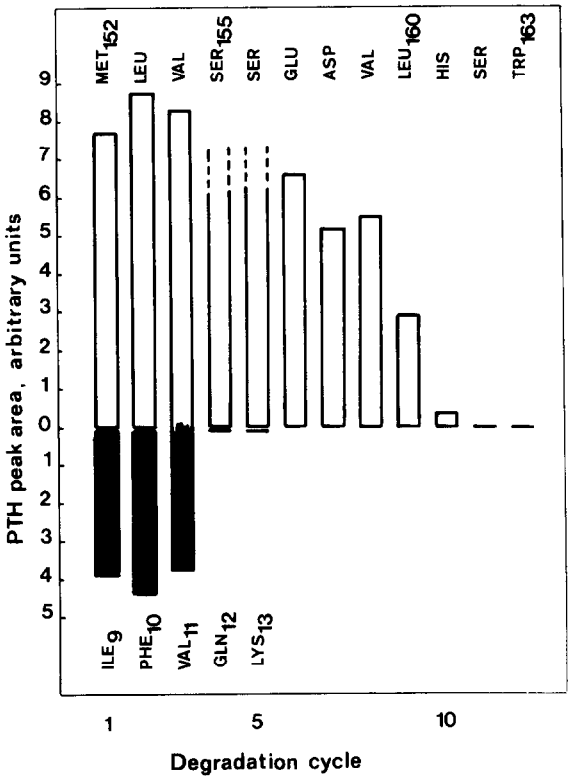

Fig.3. Edman degradation of the subunit II–cytochrome *c* crosslinked tryptic peptides: (□) and (■) PTH amino acid derivatives area after HPLC, obtained in each cycle, from subunit II and cytochrome *c*, respectively. In the third step the residues from subunit II and cytochrome *c* crosslinked peptides coincide. The quantitation of Ser in cycle 4 and 5 cannot be done because of the decomposition of anilinothiazolinone of this amino acid during conversion. For details see text.

fragment decreased suddenly on cycle 4 at the position of glutamine 12, one residue before the crosslinked amino acid, lysine 13. This was not due to washing of the crosslinked product from the cup because there was no great decrease in yield of residues from the subunit II fragment. It must instead represent a steric effect. There was a sudden drop in the degradation yield of the subunit II fragment on cycle 10 at the position of histidine 161. This residue and Trp 163, both potent electron donors, are therefore the moste likely targets of the electrophilic attack of the highly reactive nitrene.

These results locate one site of cross linking of arylazido-cytochrome *c* (modified at lysine 13) within subunit II at or close to histidine 161. This residue is conserved in subunit II of cytochrome *c* oxidase from various sources (table 1) and might be a candidate as ligand to redox centers in the enzyme. Moreover in the crosslinked peptide, there are two close-by negatively charged amino acids (glutamic acid 157 and aspartic acid 158) one of which, Asp 158, is also conserved and it can be inferred as the site of interaction of unmodified lysine 13.

The above conclusions are based on analysis of 1 of 4 heme-containing fractions generated by cleavage of the cytochrome *c*–subunit II crosslinked product. The other 3 peaks may contain the same crosslink but with either the cytochrome *c* portion or subunit II portion involving larger fragments not fully degraded in the proteolysis step. However, until these other heme-containing peaks have been fully analysed we cannot rule out the possibility of other linkage sites on subunit II for the arylazido-cytochrome *c* derivative.

Our results to date establish that the C-terminal portion of subunit II is involved in cytochrome *c* binding and must be on the C domain on the cytoplasm side of the membrane. This part of subunit II also contains 2 cysteines and other amino acids thought to ligand a copper atom and possibly 1 of the heme prosthetic groups [12–15]. The localization of the C-terminus of subunit II on the C domain is consistent with the folding pattern for this polypeptide in the cytochrome *c* oxidase complex [16].

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